Allelic Loss of a Common Microsatellite Marker *MYCL1*: A Useful Prognostic Factor of Poor Outcomes in Colorectal Cancer

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ABSTRACT

Purpose: **Allelic loss involving chromosome arms 5q, 8p, 17p, and 18q is commonly detected in colorectal cancer (CRC). The short arm of chromosome 1 is also frequently affected in a whole range of cancer types, including CRC. Our aim in the present study was to determine whether allelic losses on 1p were likely to be of much value in predicting the prognosis of CRC cases.**

Experimental Design: **Genomic DNA was prepared from tumor and corresponding normal tissue specimens from 90 patients who had undergone curative resection for CRC. Loss of heterozygosity (LOH) on chromosome arms 1p, 2p, 5q, 7q, 8p, 17p, 17q, and 18q was examined using 14 microsatellite markers, and possible correlations between LOH and clinicopathological factors (including tumor recurrence and patient survival) were investigated. LOH at the *MYCL1* microsatellite marker at 1p34 was detected in 12 of 74 (16.2%) patients who were informative for this marker.**

Results: **After controlling for tumor stage and gender and excluding findings for patients with remote metastasis, we found that patients who were positive for LOH at *MYCL1* were 31 times more likely to experience recurrence than those who were negative for LOH at this locus (95% confidence intervals, 2.27-∞; $P = 0.04$). There were indications of a similar tendency for LOH at the *14-3-3-σ-TG* microsatellite marker at 1p35, but we could find no evidence of a significant association between LOH at this site and tumor recurrence or patient survival. We were also unable to detect significant association between LOH at the various sites on 2p, 5q, 7q, 8p, 17p, 17q, and 18q and either tumor recurrence or patient survival.**

Conclusions: **CRC patients whose tumors exhibited LOH at *MYCL1* at chromosome 1p34 were likely to have a poor prognosis, suggesting that this marker may have clinical relevance.**

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent malignancies in developed countries. The clinical outcome of CRC patients with stage I and II disease is generally good after curative surgery (1, 2) but is more likely to be adverse in patients with stage III and IV disease despite radical resection and adjuvant chemotherapy. Although tumor stage is the most useful prognostic factor (1, 3), it is not nearly as useful as a predictor of recurrence. Empirically, clinicopathological factors other than stage, such as age and tumor differentiation, are considered useful, but they do not always provide enough information upon which to base predictions of patient outcome (1, 4–6). Considerable effort has therefore been expended in defining the molecular and biological mechanisms associated with tumor development and progression. The relatively well-defined stages through which CRC develops have facilitated the discovery of inherited and acquired gene and chromosome defects that contribute to the tumor phenotype (7–9). Activation of oncogenes such as K-*ras* or deletion of tumor suppressor genes, including *APC* on 5q, *p53* on 17p, and a region of chromosome 18q that includes the *DCC* and/or *DPC4* genes, may also be important in the progression of CRC (10–22). Among chromosome defects, deletions in 17p and 18q are said to be the most frequent late-stage events (14, 20–22). A recent report indicates that allelic imbalance of 8p may be useful in CRC prognosis (23).

Alterations to the short arm of chromosome 1 are also fairly frequent in CRC, and several authors have claimed that allelic loss on 1p may be of some use as an independent predictor of poor outcome in patients with a variety of cancers and in some CRCs (14, 15, 17, 19). These findings suggest that there could be a tumor suppressor gene of relevance to cancer progression on 1p, but no specific gene whose allelic loss contributes to the development of CRC and/or other cancers has yet been identified on this arm of the chromosome (24–26). In the present study, we simply attempted to determine whether the presence or absence of allelic loss on *MYCL1* located at 1p34 is likely to be of prognostic value in CRC.

MATERIALS AND METHODS

Patients and Tumor Specimens. Paired primary tumor and normal tissue specimens from 90 CRC patients with cura-

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tive treatments were obtained from the Department of Surgery, Okayama University Medical Hospital and the Okayama Saiseikai Hospital. All patients had undergone curative surgery between 1994 and 1999; they received no chemo- or radiotherapy before surgery. Tumors had to be large enough to provide sufficient tissue for genetic analysis without compromising pathological diagnosis. Tissues from nonnecrotic areas of the tumor and normal mucosa were placed on ice immediately after removal from the patient and frozen at −80°C until DNA extraction commenced. In prognosis analysis, all of the CRC patients with curable resection were enrolled. Potential confounders of relationships between loss of heterozygosity (LOH) and prognosis were measured and controlled in the analysis when necessary. The following factors were considered: age; gender; adjuvant chemotherapy; tumor stage; and the degree of carcinoma differentiation.

Clinical Characteristics. The data collected from patient records included age, gender, date and type of surgery and/or adjuvant chemotherapy, tumor differentiation, lymphovascular invasion, stage based on tumor-node-metastasis (TNM) classification, recurrence status, total length of follow-up, and disease status at last follow-up. All cases were assigned an identification number, and patient confidentiality was maintained. Treatment of stage I and II tumors mainly involved only surgical resection. 5-Fluorouracil-based adjuvant chemotherapies were used in patients with stage III and IV tumors. Follow-up data were confirmed by chart and pathological report review.

LOH Analysis. DNA from paired normal and tumor tissues was examined for LOH with respect to two microsatellite markers on the short arm of chromosome 1. *MYCL1*, a tetranucleotide repeat located upstream of *L-Myc* on 1p34, and *14-3-3-σ-TG*, a TG repeat sequence in the 3′-untranslated region of *14-3-3-σ* on 1p35, were used to assess LOH on chromosome 1p (27). Markers used for the analysis of other regions were *D1S211* (1p34), *D1S228* (1p36), *D2S123* (2p16), *D5S107* (5q11–13), *D5S346* (5q21–22), *D7S480*, *D7S486*, *D7S522* (7q31), *D8S87* (8p12), *D8S254*, *D8S258* (8p22), *D17S250* (17p11–12), *D17S261* (17q11–12), *D17S960* (17pter-qter), *D18S35* (18q21), and *D18S58* (18q22–23). PCR was carried out in a 50-μl reaction mixture consisting of 100 ng of genomic DNA, 0.2 μM of each oligonucleotide primer pair (one end-labeled with Texas Red), 200 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 5 μl of 10× PCR buffer, and 1.25 unit of Taq polymerase (Ampli-TaqGold; Perkin-Elmer, Foster City, CA). Cycling conditions were 95°C for 10 min, followed by 35 cycles of 94°C for 30 s, the specified annealing temperature 72°C for 30 s, with a final extension step of 7 min at 72°C. After denaturation in formaldehyde at 95°C for 5 min, the amplified PCR products were electrophoresed on a 6% Long-Ranger-6.1 M urea gel on Hitachi Autosequencer SQ-5500 and analyzed by FRAGRYS version 2 software (Hitachi Inc., Tokyo, Japan). Informativity was defined by the presence of two visible alleles in normal tissue DNA. Tumors whose PCR products showed microsatellite instability (MSI) were excluded from LOH analysis of the various loci. When the signal intensities of alleles of tumor DNAs were compared with those of corresponding normal DNAs, a reduction of at least 50% in signal intensity was taken as indicative of LOH. All LOH results were interpreted blind before matching to the clinical status of the patient involved.

Statistical Analysis. Statistical end points in our analysis were disease-free survival from the date of curative surgery and CRC recurrence status at 36 months. Survival analysis was conducted with censoring of patients who died from other diseases or showed no evidence of recurrence. The probability of disease-free survival was plotted over time using the Cox proportional hazards model, and differences between groups were tested with maximum likelihood statistics, adjusting for potential confounders. Logistic regression and Cochran-Mantel-Haenszel statistics were used to calculate odds ratios (ORs) of recurrence at 36 months with 95% confidence intervals. Factors that were significantly related to both LOH status and recurrence in the univariate analysis were entered into the multivariate analysis. Descriptive or stratified analysis always preceded parametric modeling to confirm that the assumptions underlying the models were met. The results are reported as two-sided maximum likelihood *P*s. Analyses were performed using the Statistical Analysis System (SAS) Version 8.0 (SAS Institute Inc., Cary, NC).

RESULTS

Preliminary analysis of various microsatellite markers in 72 CRC patients demonstrated that patients whose tumors were positive for 1p (*MYCL1*) LOH were about 10 times as likely to experience a recurrence as patients whose tumors were LOH negative ($P = 0.03$), controlling for tumor stage, age, and gender. Tumor recurrence did not appear to be significantly associated with LOH at any of the non-1p markers that we tested.[4]

Further analysis was therefore limited to possible relationships between 1p alteration and clinical outcome in a large number of CRC patients. We studied a total of 90 patients with curative operations for CRC, all of whom were subjected to detailed analysis including careful LOH analysis of *MYCL1* at 1p34 and *14-3-3-σ-TG* at 1p35.

In characterizing the 25 patients whose cancers recurred within 36 months, we noted that the time from diagnosis to the onset of recurrent disease was a mean of 20.0 months (SD = 10.6 months). There were 61 patients who either remained disease free for 36 months or experienced a recurrence during this time. We therefore used disease-free survival at 36 months as the key study outcome because most patients had been followed for at least this long, and all but one recurrence had been detected within this time frame. One patient experience disease recurrence at 39 months and was treated as a nonrecurrence. Classifying this patient as nonrecurrent in our analysis did not affect the results.

Table 1 shows the demographics of 61 CRC patients with recurrence status at 36 months. Gender, chemotherapy, and stage were all related to recurrence and hence were all considered in the analysis. Among the 90 patients examined, 74 were informative for the *MYCL1* marker, and 16.2% showed LOH.

[4] T. Nagasaka, G. B. Sharp, and N. Matsubara, unpublished observations.

Table 1 Demographics and other characteristics of colorectal cancer patients recurring within or followed for at least 36 months[a]

	Recurrence				
	No		Yes		
Characteristic	No	%	No.	%	P^b
Gender					
Female	16	88.9	2	11.1	
Male	20	46.5	23	53.5	0.004[b]
Age at diagnosis					
<50	4	44.4	5	55.6	
50–59	6	54.6	5	45.5	
60–69	18	58.1	13	41.9	
70–79	6	75.0	2	25.0	
80<	2	100.0	0	0	
Mean (years)	64.2		59.3		0.06[c]
Adjuvant chemotherapy					
No	22	78.6	6	21.4	
Yes	14	42.4	19	57.6	0.008[b]
Differentiation of carcinoma					
Well	10	83.3	2	16.7	
Moderate	26	54.2	22	45.8	
Poor	0	0	1	100.0	0.09[d]
Tumor location					
Proximal	10	58.8	7	41.2	
Distal	26	59.1	18	40.9	1.0[b]
Stage					
I	11	100.0	0	0	
II	12	70.6	5	29.4	
III	12	52.2	11	47.8	
IV	1	10.0	9	90.0	0.0001[d]

[a] One patient who recurred at 39 months is counted as a non-recurrence in this analysis.
[b] Fisher exact two-tailed test.
[c] Two-tailed *t* test.
[d] Chi-square test.

Table 2 shows the characteristics of the 74 CRC patients according to their LOH status at *MYCL1*. Although the LOH status of *MYCL1* and *14-3-3-σ-TG* were strongly correlated, LOH at *MYCL1* was not significantly associated with any other factors in the univariate analysis.

Of the 25 patients with curative treatments who experienced recurrence within 36 months, 13 (52%) had disease recurrence within 5–16 months, the other 12 (48%) experienced disease recurrence in 19–39 months. We classified these patients as early and late recurrence patients, respectively (Table 3). Gender and chemotherapy were primarily associated with later recurrence as opposed to early recurrence. Late-stage disease was primarily associated with early recurrence, although stage IV disease was significantly associated with both early and late recurrence. Deletion of *MYCL1* also appeared to be significantly associated with both early and late recurrence, suggesting that it might be of value as a prognostic factor in much the same way as tumor stage has been used. LOH at *14-3-3-σ-TG* did not appear to be significantly associated with either early or late recurrence, despite the fact that *14-3-3-σ-TG* LOH and *MYCL1* LOH were intercorrelated and OR point estimates for *14-3-3-σ-TG* exceeded the null value (1.0).

Table 4 presents the crude and adjusted ORs of recurrence at 36 months for LOH at *MYCL1* and *14-3-3-σ-TG* separately. The *P*s shown here were taken from the adjusted, multivariate logistic models. Patients with LOH at *MYCL1* were at about a 31-fold increased risk of recurrence within 36 months of surgery ($P = 0.04$), taking gender and stage into account. The 95% confidence interval for the adjusted OR was 2.27-∞. Patients with deletions of *14-3-3-σ-TG* were less likely to experience recurrence than patients with *MYCL1* deletions. Although the 95% confidence interval for the crude OR for LOH at *14-3-3-σ-TG* excluded the null value, the confidence interval for the adjusted OR did not. The results obtained when the two 1p deletions were considered together are shown in Table 4. Patients with deletions of either *MYCL1* or *14-3-3-σ-TG* or both were significantly more likely to experience disease recurrence (after controlling for gender and stage) than were those in whom there was no evidence of LOH for either marker ($P = 0.02$).

As shown in Figs. 1 and 2, deletion of *MYCL1* was significantly associated with recurrence ($P = 0.01$) and death ($P = 0.03$) when gender and stage were taken into account. Male patients were more likely to experience both outcomes, but this was not statistically significant. (Taking gender out of survival models had little effect on the relationship between *MYCL1* and recurrence or death.) Deletions of *14-3-3-σ-TG* did not appear to be significantly associated with either recurrence or death when stage and sex were included in the model.[4] We also studied the LOH of other microsatellite markers close to *MYCL1* and *14-3-3-σ-TG* including *D1S211* (1p34) and *D1S228* (1p36) and correlated it with recurrence or death of the patients. Again, LOH at *MYCL1* was the strongest prognostic indicator among the four markers.[4]

DISCUSSION

This study demonstrates for the first time that LOH at *MYCL1* (1p34) is strongly related to both recurrence and sur-

Table 2 Characteristics of patients with colorectal cancer, according to LOH status of *MYCL1*[a]

Characteristic	*MYCL1 LOH*[b] *(−)* No. (%)	*MYCL1 LOH (+)* No. (%)	P^b
Age			
≤65	33 (82.5)	7 (17.5)	
>65	29 (85.3)	5 (14.7)	1.0
Sex			
Female	18 (85.7)	3 (14.3)	
Male	44 (83.0)	9 (17.0)	1.0
Chemotherapy			
No	34 (85.0)	6 (15.0)	
Yes	28 (82.4)	6 (17.6)	0.76
Stage			
I–II	26 (86.7)	4 (13.3)	
III	29 (85.3)	5 (14.7)	1.0
IV	7 (70.0)	3 (30.0)	0.34
Differentiation of carcinoma			
Well	13 (92.9)	1 (7.1)	
Moderate or poor	49 (81.7)	11 (18.3)	0.44
14-3-3-σ-TG			
LOH (−)	28 (100)	0 (0)	
LOH (+)	2 (20.0)	8 (80.0)	<0.0001

[a] Analysis includes all patients who did not recur, regardless of time followed, as well as all patients who experienced recurrences.
[b] LOH, loss of heterozygosity.
[c] Fisher exact two-tailed test.

Table 3 Early versus late recurrences among patients recurring within 36 months according to clinical and pathological characteristics

Characteristic	Recurrence: None ($n = 36$) No.	Early (within 1–16 months; $n = 13$) No.	Early OR[a] (95% CI)	Late (after 16 months; $n = 12$) No.	Late OR (95% CI)
Age					
≤65[a]	21	8		8	
>65	15	5	0.9 (0.24–3.25)	4	0.7 (0.18–2.79)
Gender					
Female[b]	16	2		0	
Male	20	11	4.4 (0.91–21.25)	12	20.1 (1.11–365.73)[c]
Chemotherapy					
No[b]	22	4		2	
Yes	14	9	3.5 (0.91–13.71)	10	7.8 (1.49–41.30)
Stage					
I–II[b]	23	1		4	
III	12	6	11.5 (1.23–106.85)	5	2.4 (0.54–10.61)
IV	1	6	138.0 (7.49–2542.86)	3	17.2 (1.41–210.12)
Differentiation of carcinoma					
Well[b]	10	1		1	
Moderate or poor	26	12	4.6 (0.53–40.28)	11	4.2 (0.48–37.17)
MYCL1					
LOH (−)	27	5		7	
LOH (+)	1	4	21.6 (1.97–235.74)	4	15.4 (1.48–160.76)
14-3-3-σ-TG					
LOH (−)[b]	16	4		2	
LOH (+)	4	5	5.0 (0.90–27.68)	2	4.0 (0.42–37.78)

[a] OR, odds ratio; CI, confidence interval.
[b] Referent.
[c] Logit confidence limits were calculated because of zero cell.

vival in CRC patients. Tumor recurrence appears to be the most significant factor in determining patient prognosis. In this study, we dealt with the data for patients who experienced recurrence in two separate groups, namely, those experiencing early recurrence and those experiencing late recurrence. This was felt to be appropriate because about half of the patients had experienced recurrence within 5–16 months of surgery, whereas the other half experienced theirs within the 19–39-month period. Factors related to recurrence, including gender and chemotherapy, were mostly associated with a later recurrence rather than an early one. By contrast, stage appeared to be significantly associated with both early and late recurrence; this indicates that stage is likely to be suitable as a prognostic factor. Deletion at *MYCL1* was also significantly associated with both early and late recurrence, suggesting that this too might be a useful prognostic factor. This is confirmed by our finding that patients who had experienced LOH at *MYCL1* turned out to have a 31-fold increased risk of recurrence by 36 months, after controlling for gender and stage. Although LOH at *MYCL1* and *14-3-3-σ-TG* appeared to be strongly correlated, LOH of either allele did not

Table 4 Odds ratios for colorectal cancer recurrence of stage I–IV patients at 36 months associated with deletions of the 1p genes[a]

Status	Recurrence at 36 months: No No. (%)	Yes No. (%)	Crude odds ratio (95% CI)[b]	Adjusted odds ratio (95% CI)	*P*
MYCL1					
LOH (−)[c]	27 (69.2)	12 (30.8)			
LOH (+)	1 (11.1)	8 (88.9)	18.0 (2.99–108.44)	30.8 (2.27–∞)[d]	0.04[d]
14-3-3-σ-TG					
LOH (−)[c]	16 (72.7)	6 (27.3)			
LOH (+)	4 (36.4)	7 (63.6)	4.7 (1.02–21.37)	8.6 (0.86–169.80)[e]	0.09[e]
Deletion of neither[c]	16 (80.0)	4 (25.0)			
Deletion of either or both	4 (30.8)	9 (69.2)	9.0 (1.92–42.25)	28.4 (2.58–∞)[e]	0.02[e]

[a] One patient recurring after 36 months counted as non-recurrence.
[b] CI, confidence interval; LOH, loss of heterozygosity.
[c] Referent group.
[d] Adjusted for gender and stage.
[e] Adjusted for stage only.

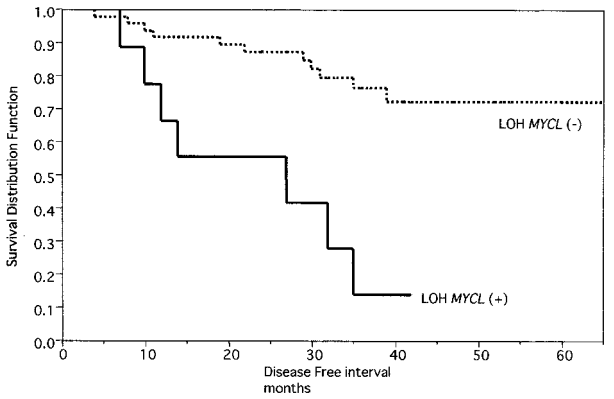

Fig. 1 Recurrence-free survival of colorectal cancer patients by loss of heterozygosity status of the *MYCL1* marker. Controlling for stage and gender, the hazard ratio for loss of *MYCL1* was 3.2 (95% confidence interval, 1.28–8.28; $P = 0.01$). The ratio for males *versus* females was 3.4 (95% confidence interval, 0.77–5.18; $P = 0.11$); the ratio per increased level of stage was 2.8 (95% confidence interval, 1.47–5.18; $P = 0.002$).

appear to be significantly associated with any other factor in univariate analysis. We also noted that *14-3-3-σ-TG* was likely to be much less useful than *MYCL1* in predicting recurrence or survival in CRC patients.

It is important to identify prognostic factors that may help to determine the grade of malignancy to provide accurate predictions of postoperative recurrence risk and guide adjuvant therapy for patients with CRC. Previous investigators have examined the value of a variety of genetic alterations as prognostic markers in CRC, including mutations of K-*ras*, *p53*, and *DCC* as well as allelic loss at 1p, 5q, 8p, 17p, and 18q. We first analyzed K-*ras* mutations and looked for LOH on chromosome arms 2p, 5q, 7q, 8p, 17p, 17q, and 18q, but we found no significant association between any of these factors and either recurrence or survival in CRC patients.

MYCL1 is a tetranucleotide repeat microsatellite marker commonly used to analyze the status of MSI and is among the frequently mutated markers used to identify low-level MSI (28–30). In cases where a sample does not show MSI, it turns out that *MYCL1* is likely to be a very useful marker for LOH analysis, largely because of the high frequency with which it is found to be informative. This being the case, it may be that a large amount of data of relevance to the LOH status of *MYCL1* in CRC has already been gathered by investigators in a wide variety of institutions. Our data indicate that systematic reanalysis of the *MYCL1* status of CRC patients will almost certainly be able to provide clinicians with significant information about the most likely patient outcomes. We believe that a future large-scale collaborative study will allow our results to be confirmed or contradicted as a result of much more systematic and/or definitive trial.

Associations between allelic loss on various sections of 1p and clinical outcomes in CRC have been described in other studies. Thus, for example, Ogunbiyi *et al.* (17) showed that allelic loss at 1p32 and 1p36 was significantly associated with poor prognosis in 116 patients with stage I–III disease. In our study, an unfavorable outcome was significantly associated with allelic loss of microsatellite markers at *MYCL1* and less significantly associated with allelic loss at other markers located close to *MYCL1*.[4]

It is known that only a very few non-mononucleotide microsatellite markers show frequent mutation outside high-level MSI cancers. One of these is the tetranucleotide marker *MYCL1*. It is possible that frequent finding of *MYCL1* mutation is selected by virtue of a functional effect that is advantageous to the lesion. There is evidence that *p53* regulates the *PIG3* promoter at a pentanucleotide repeat and that different polymorphisms in this repeat influence transcription of *PIG3* (31). Thus, alteration of *MYCL1* might conceivably reduce transcription of an unknown gene. Accordingly, loss of the wild-type copy of *MYCL1* might then have a tumorigenic and prognostic effect. Although *MYCL1* mutation and 1p LOH in the same tumor are difficult to demonstrate, six tumors with MSI in *MYCL1* and 1p LOH in nearby loci revealed unfavorable prognosis.

The net result is that LOH at *MYCL1* in CRC patients may well prove to be a very valuable (and practical) prognostic marker that can be used to supplement the more traditional clinicopathological staging methods that are currently being used for CRC patients. It is interesting that four of five stage II tumors that recurred showed *MYCL1* loss, and *MYCL1* loss in stage II tumors may be of more clinical importance. Nevertheless, any significant clinical application of our findings will need to await confirmation of the results obtained with our small cohort in either a series of independent studies or probably one or more large-scale collaborative studies.

To summarize, allelic loss at *MYCL1* is likely to be a useful prognostic factor in patients with CRC who have undergone curative surgery. Thus, for example, CRC patients with available preoperative biopsy samples who showed LOH at *MYCL1* may well benefit from extended curative surgery. Identification of LOH at this site should be a more effective means of separating patients into those whose risks of recurrence and death are high or low.

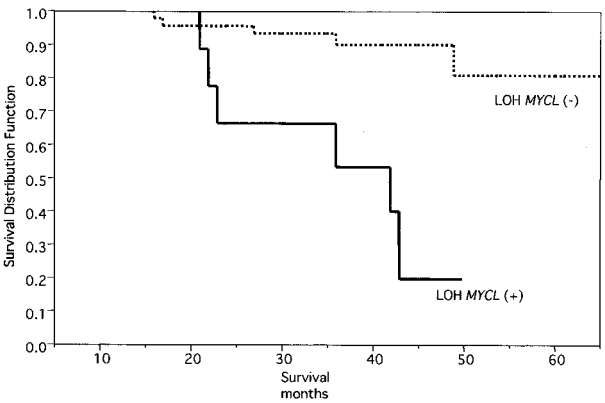

Fig. 2 Overall survival of colorectal cancer patients by loss of heterozygosity status of the *MYCL1* marker. Controlling for stage and gender, the hazard ratio for loss of *MYCL1* was 4.0 (95% confidence interval, 1.13–14.39; $P = 0.03$). The ratio for males *versus* females was 2.3 (95% confidence interval, 0.28–14.81; $P = 0.44$); the ratio per increased level of stage was 3.1 (95% confidence interval, 1.21–7.81; $P = 0.02$).

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